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Chloroplast transformation by *Agrobacterium tumefaciens*

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A chimeric gene consisting of the promoter region of the nopaline synthase gene (*Pnos*) fused to the coding sequence of the chloramphenicol acetyltransferase gene (*cat* gene) of Tn9 was introduced by co-cultivation in tobacco protoplasts followed by selection with 10 μg/ml chloramphenicol. The chloramphenicol-resistant plants derived from these selected calli were unable to transmit the Cm^R phenotype through pollen. A typically maternal inheritance pattern was observed. Southern blot analysis showed that the chimeric *Pnos-cat* gene was present in the chloroplasts of these resistant plants. Furthermore, the chloramphenicol acetyltransferase activity was shown to be associated with the chloroplast fraction. These observations are the first proof that the *Agrobacterium* Ti-plasmid vectors can be used to introduce genes in chloroplasts.

Key words: *Agrobacterium*/chloroplast transformation/Ti-plasmid vectors/chimeric genes

Introduction

The Gram-negative soil bacterium *Agrobacterium tumefaciens* can transfer DNA to a variety of plants. All cases studied thus far have indicated that the transferred bacterial DNA-segment (T-DNA) is located and expressed in the nucleus of the transformed plant cells (Willmitzer *et al.*, 1980; Chilton *et al.*, 1980; for a review, see Caplan *et al.*, 1983). However in all these instances the transferred genes, whether they were derived from the wild-type Ti-plasmid or consisted of experimentally constructed genes introduced in plants *via* Ti-plasmid-derived vectors, are under the control of nuclear transcription initiation sequences (Herrera-Estrella *et al.*, 1983a,1983b; Fraley *et al.*, 1983; Bevan *et al.*, 1983; Horsch *et al.*, 1984; De Block *et al.*, 1984).

Experimental evidence indicates that transcription initiation signals involved in gene expression in chloroplasts are different from those needed for nuclear gene expression (Whitfeld and Bottomley, 1983; Kong *et al.*, 1984; Link, 1984; Poulsen, 1984; Crossland *et al.*, 1984). It was, therefore, still an open question whether or not *Agrobacterium* Ti-plasmid-mediated DNA transformation could also result in DNA uptake by chloroplasts, since selection for transformed plant cells thus far relied on nuclear expressed genes and not on genes designed to function in chloroplasts.

Here we report the first observations indicating that when a Ti-plasmid vector contains a marker gene, capable of being expressed in chloroplasts, genetically transformed chloroplasts can be detected in plant cells transformed by *Agrobacterium*.

Results

Maternal inheritance of a chloramphenicol acetyltransferase gene

The first indication that transformation of chloroplasts may occur after Ti-plasmid-mediated DNA transfer, resulted from an analysis of plants transformed with the chimeric marker gene P*nos-cat*. This gene consists of the promoter region of the nopaline synthase gene fused to the coding sequence of chloramphenicol acetyltransferase (*cat*) of Tn9 (see Figure 1a) (De Block *et al.*, 1984). Using pGV3850:pNCAT 7 as a Ti-plasmid vector in co-cultivation experiments with protoplasts of *Nicotiana tabacum* cv. Petit Havana SR1, calli resistant to 10 μg/ml of chloramphenicol could be selected. As described earlier (De Block *et al.*, 1984), the chloramphenicol (Cm)-resistant calli were of two types: 5% carried the Nos marker, whereas 95% were Nos negative. Figure 1b illustrates the formation in *Agrobacterium* of a hypothetical intermediate that is transferred to the plant cell where it integrates in the genome. This model would explain the occurrence of the Cm^RNos^- transformants. Evidence for the occurrence of such an intermediate was presented earlier (De Block *et al.*, 1984).

All the subsequent results which we describe here were obtained with material derived from Cm^RNos^- calli. Twenty plantlets regenerated from such calli were tested for their resistance to chloramphenicol by the ability of stem fragments to root on chloramphenicol-containing medium (De Block *et al.*, 1984). Both chloramphenicol-sensitive (12 out of 20) and chloramphenicol-resistant (8 out of 20) plants were obtained from the same callus.

Enzymatic assays demonstrated that the chloramphenicol-resistant plants contained Cat activity whereas the sensitive plants were devoid of this activity. Southern blot hybridizations using a radioactively labelled *cat* probe (Figure 2) confirmed that the Cm-resistant plants contained the *cat* gene whereas the Cm-sensitive plants did not hybridize with the *cat* probe, indicating that the latter plants regenerated from cells that had lost the *cat* gene. One possible explanation for the frequent loss of chloramphenicol resistance would be a cytoplasmic location of the transferred *cat* gene. To test this possibility a Cm^R plant (rGV3002) was used as a pollen donor in a cross with a wild-type *N. tabacum* cv. Petit Havana SR1 plant. Twenty-two seedlings were tested for chloramphenicol resistance and another 10 for Cat activity, and all turned out to be negative, indicating that the *cat* gene had not been transmitted in this cross. Reciprocally, the rGV3002 plant was castrated and pollinated by pollen from a wild-type SR1 plant. In this case at least 80% of the offspring seedlings were shown to exhibit Cat activity. The relative Cat activity in these different seedlings, however, varied markedly. Similar results were obtained if the seedlings of a selfed rGV3002 plant were tested. Thus, the Cat activity was inherited in a maternal fashion and might therefore be transmitted through cytoplasmic inheritance.

The cat gene in rGV3002 is present in the chloroplast

To explain the observed maternal inheritance, nuclear, chloroplast and mitochondrial DNA of rGV3002 was analyzed using Southern blot hybridizations (Figure 2). The presence of a *cat* gene was detected in total DNA as a 0.7-kb (Figure 2, lane 1) internal *Eco*RI fragment (see map Figure 1a) or as a 12- and 5.4-kb fragment in an *Xho*I digest (Figure 2, lane 2). There are no *Xho*I sites in the *cat* gene construct (Figure 1a). The two hybridizing *Xho*I fragments (Figure 2, lane 2) probably correspond in the chloroplast population to either two independently integrated fragments or to fragments where one is a rearranged version of the other one.

Using pNCAT 7 as a probe, no hybridization was detected in either nuclear or mitochondrial DNA (lanes 3 and 4) whereas chloroplast DNA contained DNA fragments hybridizing to the *cat* probe (Figure 2, lane 5). In the *Xho*I chloroplast pattern, the same fragments hybridizing to a *cat* probe also hybridized to the other vector-specific probes (pBR322, pTiC58 *Hind*III fragment 23, 1.1-kb *Sma*I/*Hind*III *neo* fragment of pKC 7, data not shown). There was no hybridization with purified pTiC58 *Hind*III fragment 10. No hybridization to any of the probes was detected in a chloramphenicol-sensitive control plant derived from the same original callus (Figure 2, lane 6).

The sequence of the promoter region of this construct was analysed for the presence of signal sequences that might explain why the P*nos-cat* chimeric gene can be expressed in chloroplasts. The chimeric gene still contains the Shine and Delgarno sequence derived from the bacterial *cat* gene. Furthermore, the 'ATAATT' and 'TTG' sequences derived from the *nos* promoter region indicated in Figure 1a could provide procaryotic transcription signals.

The hybridizing *Xho*I fragments (12 and 5.4 kb) in the chloroplast DNA are different in relative intensity from the supposedly equivalent fragments in the *Xho*I digest of total DNA. Since the chloroplasts used in this experiment were harvested from a subculture of the plant from which the total DNA was extracted 6 months earlier, the possibility exists that during subculturing an enrichment occurred for these chloroplasts harbouring the 5.4-kb fragment.

Chloramphenicol acetyltransferase expressed from a nuclear specific promoter

To understand why the use of the P*nos-cat* chimeric gene allowed detection of chloroplast transformants, we compared the expression of a *cat* gene under the control of a nuclear-specific promoter with the expression observed for the chloroplast-linked gene in rGV3002. *N. tabacum* cv. Petit Havana SR1 protoplasts were therefore transformed in a co-cultivation experiment with a chimeric gene consisting of the promoter region of the nuclear gene for the small subunit of ribulose biphosphate carboxylase (Rubisco) fused to the *cat*-coding sequence of Tn9. This chimeric gene was previously shown to be expressed in tobacco in a light-

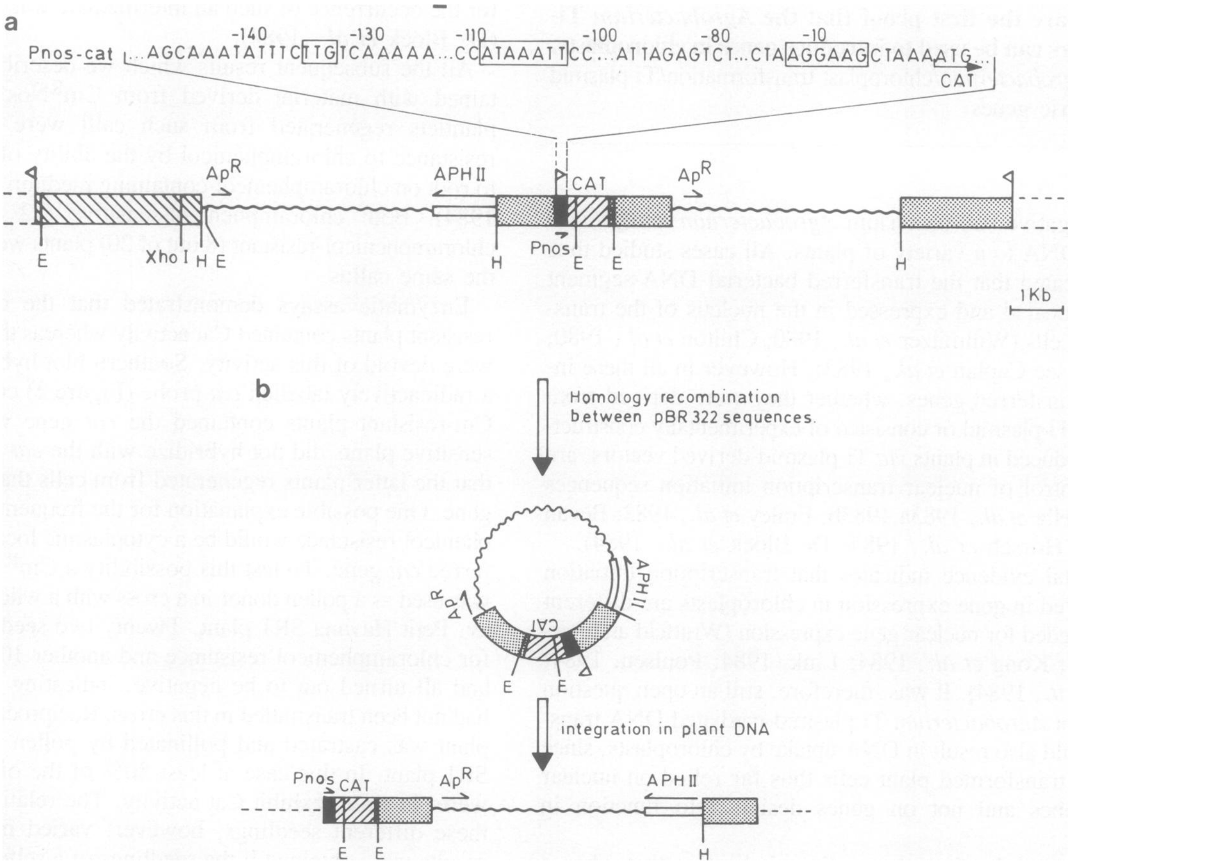

Fig. 1. (a) Restriction map of plasmid pGV3850:pNCAT 7. The sequences upstream from the initiation ATG-codon of the P*nos-cat* gene were analysed for the presence of a Shine and Delgarno sequence and for sequences which could be important for initiation of transcription in procaryotes and chloroplasts. These sequences are highlighted. ⊳ : represents the border sequences of the T-DNA (Zambryski *et al.*, 1982); ∿: pBR322 sequences; ▨: Ti-plasmid fragment *Hind*III 23 sequences; ▧ : Ti plasmid fragment *Hind*III 10 sequences; ▨: cat gene sequences. **(b)** Hypothetical model to explain the integration of the pNCAT 7 plasmid (recombined out of the original pGV3850:pNCAT 7) into the plant DNA (this can be nuclear or chloroplast DNA: represented by the dotted lines) (De Block *et al.*, 1984).

inducible manner (Herrera-Estrella *et al.*, 1984). Figure 3 illustrates the region of the vector pGV3850:pMH2 used in the co-cultivation experiment. When small calli were grown on cytokinin-containing medium to allow greening, calli resistant to 5 μg/ml but not to 10 μg/ml chloramphenicol were obtained. Greening has been shown to be a necessary condition for the induction of the *Pssu-cat* gene in tobacco (Herrera-Estrella *et al.*, 1984). When the resistant calli were transferred to media with a low cytokinin content such that they grew as white tissues, they died in the presence of 5 μg/ml of chloramphenicol.

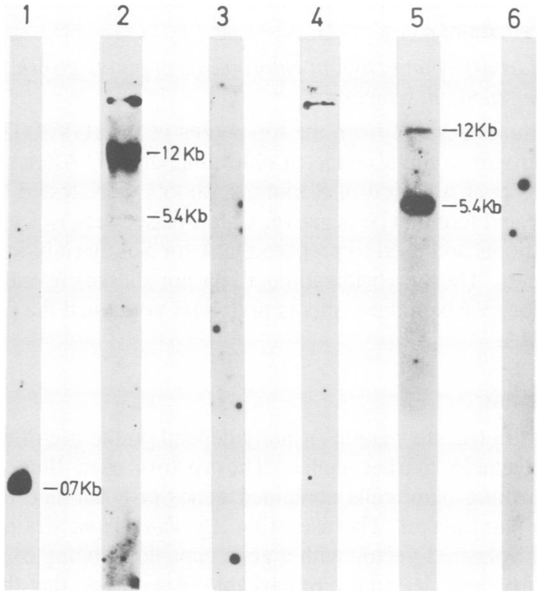

Fig. 2. Southern blot hybridization analysis of DNA prepared from the plant rGV3002. 10 μg of total DNA digested with either *Eco*RI **(lane 1)** or *Xho*I **(lane 2)** were hybridized to a purified 0.9-kb *Eco*RI fragment with the chloramphenicol acetyltransferase sequence from Tn9 (Marcoli *et al.*, 1980). **Lane 3** represents the hybridization of 10 μg nuclear DNA (cut with *Xho*I) to the ^{32}P-labelled pNCAT 7 plasmid. **Lane 4** represents the hybridization of 3 μg of mitochondrial DNA (cut with *Xho*I) with the pNCAT 7 probe. 2 μg of chloroplast DNA were digested with *Xho*I and hybridized to the 0.9-kb Cm-fragment **(lane 5)**. **Lane 6** represents the hybridization of 10 μg total DNA of a Cm^s plant derived from the same callus as rGV3002 (cut with *Xho*I) with the pNCAT 7 probe. The relative migration of the hybridizing bands in the different lanes cannot be directly compared as the DNA samples were electrophoresed in different gels at different times. Alongside each lane the sizes of the hybridizing bands are given in kb, to facilitate comparison of the bands. The hybridizing bands observed at the top of **lanes 2** and **4** are interpreted as non-specific hybridization at the slot position due to the capturing of the probe by remaining polysaccharides and/or proteins.

Plants were regenerated from the Cm^R calli and five such plants were studied in more detail. Cat activity was detected in the leaves of these plants (Figure 4). Nuclear, mitochondrial and chlorolast DNA was prepared from these different plants and hybridized in Southern blot hybridizations to probes covering the entire T-DNA sequence of the pGV3850:pMH2 vector. Hybridizations were observed with nuclear DNA only (data not shown). Figure 3 summarizes the results obtained with one of these plants (rGV3003).

This plant contains ~20 copies of the pGV3850::pMH2-T-DNA. Selection for Cm resistance repeatedly resulted in the isolation of tissues carrying multiple inserts. When P*nos-neo* or P*nos-mtx* constructs are used in selections (De Block *et al.*, 1984), the resistant tissues carry only one or a few copies of the chimeric genes (De Block *et al.*, 1984; De Block, unpublished results). These results indicate that a limited number of copies of the *cat* gene in the nucleus does not convey a convenient selectable chloramphenicol resistance to plant cells. This might explain why chloroplast transformants were readily detected in a co-cultivation experiment using the P*nos-cat* chimeric gene.

Assay for Cat activity in chloroplasts

The previous experiments proved that the P*nos-cat* gene is present in the chloroplast genome of rGV3002. Hence we expect that the Cat activity is located in the chloroplast of this plant. Intact chloroplasts were prepared from the nuclear-transformed plant rGV3003, and from the chloroplast-transformed plant rGV3002. Cat-activity was assayed both in total extracts and in purified chloroplasts. Figure 4 shows that the Cat activity in rGV3002 was associated with the chloroplast fraction whereas no Cat activity was associated with the chloroplasts of rGV3003.

Expression of a procaryotic neo gene in chloroplasts

As can be seen in Figure 1a the pGV3850:pNCAT 7 vector harbours the procaryotic neo gene (Km^R) from Tn5. Since chloroplast genes can be expressed in *E. coli* (Whitfeld and Bot-

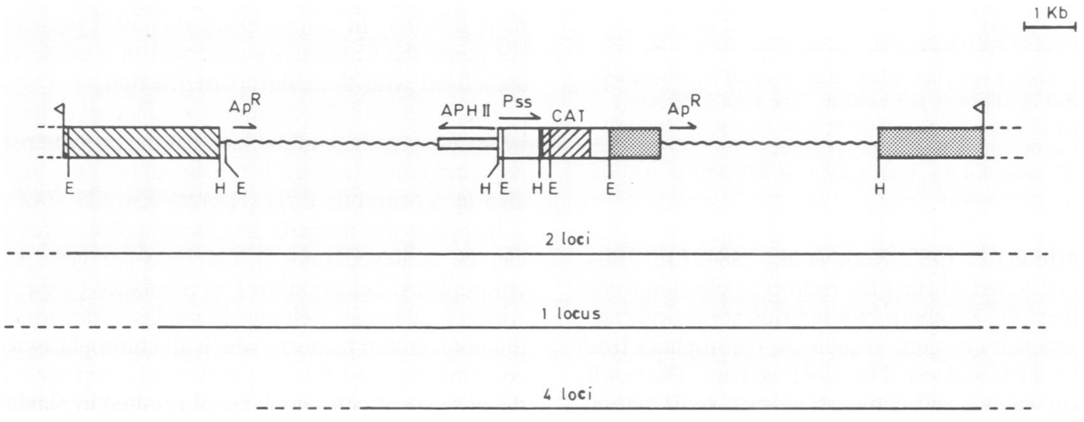

Fig. 3. Restriction map of the T-DNA of the plasmid pGV3850:pMH2. pMH2 was constructed as described by Herrera-Estrella *et al.* (1984). pMH2 was transferred from *Escherichia coli* to *Agrobacterium* by the method of Van Haute *et al.* (1983), and co-integrates with the non-oncogenic acceptor Ti-plasmid pGV3850 (Zambryski *et al.*, 1983) where selected on kanamycin-containing medium. The full lines under the restriction map represent the integrated copies of the T-DNA of pGV3850:pMH2 in the nuclear DNA of the plant rGV3003. The number of the independent loci where they integrated are indicated. However, in each locus the integrated DNA is tandemly repeated. There are ~20 copies altogether of the chimeric P*ssu-cat* gene in rGV3003. P$_{ss}$: promoter of the gene coding for the small subunit of ribulose biphosphate carboxylase. For the legend see Figure 1a.

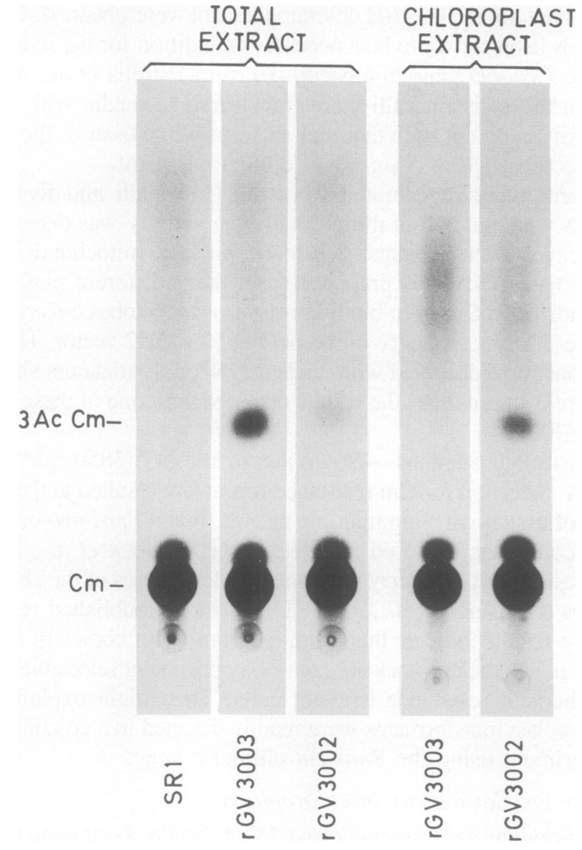

Fig. 4. Localisation of Cat activity in the chloroplasts of rGV3002. Cat activity was determined in total leaf extracts and in purified intact chloroplasts from the plants rGV3002 and rGV3003 as described in Materials and methods. The Cat assays from the total leaf extracts and from the chloroplasts were done independently so that both autoradiograms cannot be compared.

Table I. *Aph*II activity of crude extracts from leaves and purified intact chloroplasts

Plant number	Total leaf extract	Chloroplast extract
SR1	132	145
rGV 3003	104	107
3850:Neo 1	958	152
rGV 3002	241	1214

The preparation of purified intact chloroplasts and the *Aph*II enzyme assays were done as described in Materials and methods. The plant 3850:Neo 1 contains in its genome one copy of the chimeric gene P*nos-neo* (De Block *et al.*, 1984).
These numbers clearly show that the *Aph*II activity of rGV3002 is localised in the chloroplasts.

tomley, 1983; Lerbs *et al.*, 1983; Kong *et al.*, 1984; Lin, 1984; Zhu *et al.*, 1984) we tested whether the reciprocal situation would also hold true. Since the Southern blot hybridizations indicated that rGV3002 contained *neo* gene sequences, chloroplasts from these plants were isolated and tested for *Aph*II activity. The results are summarized in Table I and demonstrate that *Aph*II activity is indeed detected in chloroplasts of the rGV3002-transformed plant but not in chloroplasts of either an untransformed SR1 tobacco control or of a nuclear transformant expressing a chimeric *neo*-gene (3850 Neo 1, De Block *et al.*, 1984). In the absence of data mapping the 5′ end of the *neo* transcript obtained in the chloroplasts, we cannot at present exclude the possibility that the procaryotic *neo* gene is expressed by read-through from a chloroplast promotor. Seedlings obtained after selfing of rGV3002 or calli derived from leaf tissue from rGV3002 are only slightly more tolerant to 50 μg/ml of kanamycin than control plants.

These results explain why no transformants were previously observed using the Tn5 *neo* gene as a selectable marker for plant transformations.

Loss of transforming DNA in chloroplasts of plants grown without chloramphenicol selection

Subcultures derived from the top shoots of the rGV3002 plants were grown in the absence of chloramphenicol. After six subcultures over a period of 8 months, shoots were tested for Cat activity and found to be negative. Chloroplasts were isolated from these shoots and used to prepare DNA for Southern blotting experiments. These hybridizations (data not shown) revealed that the chloroplasts in these shoots no longer contained the pNCAT 7 - DNA.

Discussion

Successful transformations usually depend on the use of the proper selectable marker genes. The vectors used thus far for transforming plant cells contained genes programmed to function in plant nuclei. The use of an *Agrobacterium* strain carrying a Ti-plasmid vector with a gene capable of being expressed in chloroplasts, led to the remarkable observation that this efficient gene vector system can also be used to introduce genes in chloroplasts.

A chimeric gene consisting of the promoter region of the nopaline synthase gene (Herrera-Estrella *et al.*, 1983a) fused to a Tn9-derived DNA fragment coding for chloramphenicol acetyl-transferase was introduced by co-cultivation in tobacco protoplasts (De Block *et al.*, 1984) followed by selection with 10 μg/ml of chloramphenicol.

The following observations led to the conclusion that this chimeric gene is expressed when present in chloroplasts. (i) Chloramphenicol-resistant plants derived from selected calli were unable to transmit the Cm^R phenotype through pollen. A typically maternal inheritance pattern was observed.(ii) The vector DNA carrying the P*nos-cat* gene was detected in DNA from purified chloroplasts and not in nuclear or mitochondrial DNA. (iii) The chloramphenicol acetyltransferase (Cat) activity in rGV3002, one of the Cm^R plants, was shown to be associated with the chloroplast fraction. In control plant rGV3003 harbouring a nuclear *cat* gene, the Cat activity observed in a crude extract was not associated with the chloroplast fraction.

This successful demonstration of chloroplast transformation can be the consequence of the fact that the promoter sequence of the nopaline synthase gene, is functional not only in plant nuclei but also in procaryotic cells (Herrera-Estrella *et al.*, 1983b).

It appears that a significant chloramphenicol-resistant phenotype can be achieved only if the P*nos-cat* gene is expressed in chloroplasts. Selection for Cm resistance by the chimeric P*nos-cat* gene, although yielding chloroplast transformants, apparently did not result in plants in which all chloroplasts were transformed. This might well be the reason why repeated subculturing in the absence of chloramphenicol resulted in plants devoid of Cat harbouring chloroplasts.

It can be expected that new selectable marker genes specifically designed to be expressed in chloroplasts from chloroplast specific promotor sequences, will provide a stronger selection. It is hoped that the use of such chloroplast-specific genes will yield more stable chloroplast transformants.

Materials and methods

Bacterial strains and plasmids

The chimeric gene constructs were: pNCAT7 (De Block *et al.*, 1984) and P*ssu-cat* (Herrera-Estrella *et al.*, 1984). The chimeric genes were recombined into the non-oncogenic acceptor Ti-plasmid pGV3850 (Zambryski *et al.*, 1983) as described by De Block *et al.* (1984).

Plant cell culture methods

N. tabacum cv. Petit Havana SR1 was used (Maliga *et al.*, 1973). All plant cell culture methods (co-cultivation; selection for CM^R calli; shoot induction; testing for resistance of a plant by the rooting or callus induction test) were as described (De Block *et al.*, 1984). Sexual crosses were done as described by Durbin (1979).

Nopaline synthesis test

The presence of nopaline in leaf and callus material was detected as described by Aerts *et al.* (1979). To separate nopaline from the other arginine-components, chromatography on Whatman paper 540 was done instead of electrophoresis. The buffer consisted of two vol. 1-propanol to one vol. NH_4OH (25%).

Determination of chloramphenicol acetyltransferase activity

50 – 100 mg of leaf tissue was extracted by grinding manually with a glass rod in the presence of an equal volume of extraction buffer (250 mM Tris-HCl pH 7.5, 2.5 mM EDTA; 0.1% ascorbic acid; 0.5 mM leupeptine, 1 mM PMSF). Purified intact chloroplasts were prepared from 1.5 g of leaf tissue. Chloroplasts were osmotically lysed by adding 100 μl of extraction buffer to the chloroplast pellet. The mixture was heated for 10 min at 60°C. The debris were pelleted by centrifugating for 5 min in an Eppendorf centrifuge. 5 μl of 10 mM acetyl CoA and 1 μl of [^{14}C]chloramphenicol (50 mCi/mmol, NEN) were added to the supernatants. The mixtures were incubated at 37°C for 30 min, subsequently extracted with an equal volume of ethyl acetate, evaporated to dryness, and resuspended in 10 μl ethyl acetate. These samples were subjected to ascending chromatography on a silica gel thin-layer plate with chloroform/methanol (95:5) as eluant. The autoradiogram was obtained after 3 days exposure at room temperature.

Determination of AphII activity

50 – 100 mg of leaf tissue was extracted by grinding manually with a glass rod in the presence of an equal volume of extraction buffer (250 mM Tris-HCl pH 7.5, 0.1% ascobic acid, 0.5 mM leupeptine, 1 mM PMSF). Purified intact chloroplasts were prepared from 1.5 g of leaf tissue. These chloroplasts were osmotically lysed by adding 100 μl of extraction buffer to the chloroplast pellet. The debris was pelletted by centrifuging the extract for 5 min in an Eppendorf centrifuge. To a 10 μl extract, 10 μl of assay buffer (67 mM Tris-HCl pH 7.5, 42 mM $MgCl_2$, 400 mM NH_4Cl, 1.7 mM DTT), 10 μl of ATP solution (0.75 mM ATP, 20 μl [^{32}P]ATP/ml of 10 mCi/ml) and 3 μl of a kanamycin sulphate solution (1 mg/ml) were added. The reaction mix was incubated for 30 min at 37°C. The reaction was terminated by loading 30 μl of the mixture in 1 cm^2 steps onto Whatmann P-81 phosphocellulose paper. The strips were dried briefly at 68°C, washed four times at 75°C in 50 mM phosphate buffer (pH 6.5) and incubated for 45 min at 37°C in 10 mg/ml protease (Sigma, type XIV). After drying, the bound radioactivity was counted using 10 ml aquasol -2(NEN).

Preparation of intact chloroplasts

Chloroplasts were purified essentially as described by Bartlett *et al.* (1982) from plants which were kept for 1 – 2 days in the dark. To 2 g of de-ribbed leaves 10 ml of GR buffer was added (0.33 M sorbitol, 50 mM Hepes-KOH pH 7.5, 1 mM $MgCl_2$, 1 mM $MnCl_2$ 1 mM Na_2-EDTA, 1 mg/ml iso-ascorbate, 0.5 mg/ml BSA). The leaves were homogenized for 10 s at low speed (40%) in a virtis 45. The homogenate was filtered through two layers of Miracloth. The filtrate was centrifuged at 1500 *g* for 2 min. The pellet was resuspended in 1 ml of GR buffer (with a soft painting brush) and sedimented at 1500 *g* for 10 min in a continuous percoll gradient (80 – 10%).

Two bands were generated. The lower band, containing the intact chloroplasts, was carefully removed and re-suspended in 5 volumes of GR buffer. The chloroplasts were pelletted by spinning to 4300 *g* and stopping immediately (with brake off). The pellet was dissolved in the buffer used for the enzyme assay.

The preparation of nuclear, mitochondrial and chloroplast DNA

Nuclei were isolated as described by Hamilton *et al.* (1972). The DNA was isolated from the purified nuclei as described by Kislev and Rubenstein (1980).

The isolation of the chloroplast and mitochondrial DNA was essentially as described by Frankel *et al.* (1979) and Chilton *et al.* (1980).

To 8 g of de-ribbed leaves (plants were kept for 1 – 2 days in the dark), 10 ml of isolation buffer was added (0.33 M sorbitol, 5 mM $MgCl_2$-$6H_2O$, 50 mM Tris-HCl pH 7.5, 0.1% BSA, 5 mM β-mercaptoethanol). The leaves were homogenized for 10 s in a virtis 45 (at 40%). The suspension was filtered through two layers of Miracloth, and washed with an extra 15 ml of isolation buffer. The filtrate was centrifuged for 2 min at 1500 *g*. The pellet obtained consisted mainly of chloroplasts. To pellet the mitochondria, the supernatant of the first centrifugation was centrifuged at 10 400 *g* for 15 min. The pellets were resuspended in 5 ml of isolation buffer (with a soft painting brush) and brought on a discontinuous sucrose gradient (60 – 30%). The chloroplasts and mitochondria were bounded at the 60 and 30% interface by centrifuging for 30 min at 22 000 r.p.m. in a SW28 rotor.

The chloroplasts and mitochondria were taken, added to an equal volume of isolation buffer and pelletted once again at 12 000 *g* for 10 min (for chloroplasts) or for 20 min (for mitochondria). The chloroplasts were osmotically shocked by resuspending the pellet in 0.5 ml of TE buffer. The mitochondrial pellet was three times frozen with dry ice/ethanol and thawed at 22°C in a warm bath before they were resuspended in 0.5 ml TE buffer. 0.125 ml of 2% sarkosyl was added. Two phenol extractions, followed by five ether extractions were done and the DNA was precipitated with isopropanol. This yielded plastid DNA which was contaminated with 5 – 10% of nuclear DNA.

Total plant DNA preparation and genomic blottings

Plant DNA preparations were done as described by Dellaporte *et al.* (1983).

The hybridizations between the plant DNA and ^{32}P-labelled restriction fragments were done as described by Lemmers *et al.* (1980).

DNA restriction fragments were purified from agarose gels by the freeze-thaw technique (Tautz and Renz, 1983).

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